Mechanisms of Gene Therapy for Tolerance: B7 Signaling Is Required for Peptide-IgG Gene-Transferred Tolerance Induction

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LPS-activated B cells, transduced with IgG fusion proteins, are highly tolerogenic APCs. To analyze the mechanisms for this B cell-delivered gene therapy, we first followed the fate of CFSE-labeled B cell blasts. These cells primarily localized to the spleen, where a small population persisted for at least 1 mo after injection. By day 7 after injection, ~95% of the transduced cells had divided at least once, presumably an effect of the in vitro LPS activation into the cycle, because resting cells did not divide. B cells from gld donors were not tolerogenic, initially suggesting a role for Fas ligand (FasL) in tolerance. Because transduced normal B cells expressed only low levels of FasL and did not kill Fas-expressing Jurkat or A20 B lymphoma cells in vitro, these data suggest that gld B cells are not tolerogenic due to unique characteristics of these B cells rather than the lack of functional FasL expression. The transduced B cell blasts displayed significant up-regulation of both B7 costimulatory molecules, and B7.2 up-regulation was maintained through day 7 in vivo. When B cells from B7 knockout donors were transduced to express Ig fusion proteins, they were not tolerogenic in two different mouse strains and Ag models. Moreover, anti-B7 Ab blocked tolerance induction in this model, a result consistent with a role for B7 in tolerance induction. We propose that tolerance may be induced in this model by B7-driven negative regulatory signaling, but tolerance is maintained by a lack of signal 2, because expression of B7 is eventually lost in vivo.


Our laboratory has shown that IgG fusion proteins, delivered via retroviral gene therapy for B cell Ag presentation, are highly tolerogenic for the epitopes associated with the IgG (1–4). In this system, retroviral constructs have been engineered with an immunodominant epitope (e.g., p1-26 of the \( \lambda c1 \) repressor molecule) or full-length protein (e.g., OVA) in frame at the N terminus of a murine IgG1 H chain (4). B cells transduced with the fusion constructs efficiently induce both B cell and T cell tolerance in normal, immunocompetent animals (4). In the first demonstration of this, normal, immunocompetent BALB/c mice that were treated with B cell blasts expressing p12-26-IgG, were unresponsive to the peptide 12-26 at both cellular and humoral levels (4). Tolerance induction has subsequently been shown for targeted Ags in experimental models of autoimmune disease, including myelin basic protein (5), myelin oligodendrocyte glycoprotein (6), glutamic acid decarboxylase (5, 7), interphotoreceptor retinoid-binding protein (8), and clotting factor VIII (fVIII) (4) for hemophilia (9).

B cells have been demonstrated to be potent tolerogenic APCs (10–12). Previous evidence from our laboratory underscores the importance of B cells in peptide-IgG gene-transferred tolerance induction, because transduced B cell-knockout (KO) (\( \muMT \)) bone marrow failed to induce tolerance (13). Moreover, previous data suggest that the peptide-IgG fusion proteins are directly processed and presented by the transduced B cells, because MHC class II expression on the transduced cells was required for tolerance induction (13, 14).

To further clarify the mechanism of tolerance induction by peptide-IgG gene therapy, we have used CFSE to track the peptide-IgG-transduced B cell blasts in vivo. Furthermore, we have investigated the possible role of Fas ligand (FasL) and B7 expression by the B cells in tolerance induction. Our results suggest that B7 expression by the transduced B cells may be critical for tolerance induction via induction of regulatory T cells.

Materials and Methods

Virus-producer cell lines

The 12-26-IgG1 H chain cDNA was subcloned into the retroviral vector MBEA, as described previously (4). Peptide 12-26 is the immunodominant peptide from the \( \lambda c1 \) p1-102 domain in BALB/c mice. MBEA contains long terminal repeats, \( \psi^+ \) packaging signals, a neomycin resistance gene, and cloned human \( \beta \)-actin promoter sequences. Similarly, a construct encoding OVA or the entire \( \lambda c1 \) p1-102 sequence in frame with the IgG1 H chain was engineered. Virus-producer cell lines were prepared by lipofection of \( \psi^+ \) packaging cells with the engineered constructs. The cell lines were found to be helper virus free and to contain ~10\(^2\) to 10\(^3\) neomycin-resistant NIH 3T3 CFU/ml. The cell lines were cultured in RPMI 1640 (Life Technologies, Invitrogen) supplemented with 5% FBS, 2 mM L-glutamine, and 2-ME. Additionally, the cell lines were maintained in 0.6 mg/ml G418 (Geneticin; Life Technologies, Invitrogen). In one experiment, a bicistronic MSCV vector expressing a nominal Ag (domain C2 or A2 of human fVIII) (9) was also used to not only track the transduced B cells but also to validate their phenotype after in vivo transfer.

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Retroviral transduction
Splenic BALB/c or C57BL/6 B cells were infected in vitro via coculture with irradiated viral-producing packaging cells or via the addition of a filtered viral supernatant, as described previously (5, 13). B cells were purified to ~95% homogeneity with an anti-Th cell Ab mixture (anti-Th1, anti-CD4, and anti-CD8) plus complement (Low Tox M; Cedarlane Laboratories, Accurate Chemical and Scientific Corporation). Purified B cells were prestimulated with 10–25 μg/ml bacterial LPS (Escherichia coli 055: B5; Sigma) overnight and recultured with irradiated packaging cells (or viral supernatant) in the presence of 3–6 μg/ml polybrene and additional LPS for an additional 24 h.

Mice
BALB/c and C57BL/6 mice were purchased from the National Cancer Institute and from The Jackson Laboratory, respectively. Fas–deficient C57BL/6 gld and BALB/c gld mice were gifts from Dr. Wendy Davidson (Greenbaum Cancer Center, University of Maryland School of Medicine, Baltimore, MD), and IVIII KO mice on a C57BL/6 background (E16) were bred in our animal facility from a stock provided by Dr. Leon Hoyer (Hol- land Laboratory, American Red Cross). B7 KO mice (BALB/c background) were a kind gift from Dr. Arlene Sharpe (Brigham and Women’s Hospital, Boston, MA); B7 KO mice on an H-2b background were purchased from The Jackson Laboratory. All animals were used at 6–8 wk of age and housed in pathogen-free microisolator cages in our animal facility.

Tolerance induction and measurement of peptide-specific immunity
In a typical experiment, normal BALB/c recipients were given injections of gene-transferred LPS blasts (3 × 106 to 1 × 107), i.p. in a volume of 500 μl or i.v. in a volume of 100 μl serum-free medium. Five to 10 days after injection (or later), animals were immunized in a hind footpad with 25 μg of protein emulsified in CFA. Two weeks later, mice received an i.p. boost of 25 μg of protein in 500 μl of PBS. Cellular responses from the spleen were assayed 14 days after boosting. On day 3, cultures were pulsed with 1 μCi/well of [3H]thymidine (Amersham Biosciences) and incubated 16–20 h. Cells were then harvested on glass fiber filters and incorporated [3H] was detected via gas scintillation counting in a Packard Matrix 9600 reader.

CFSE staining
Cells for CFSE staining were resuspended in 1 ml of cold PBS and incubated with 5 μM CFSE (CFDA SE Cell Tracer; Molecular Probes) for 15 min at 37°C. Cells were washed extensively in serum-free medium before injection.

Abs and FACS analysis
The following Abs were purchased from PharMingen: α-CD19-PE (1D3), α-CD3-PE (16-10A1), α-CD8-PE (GL1), α-Fasl-PE (MFL3), α-B220-FITC (RA3-6B2), and α-I-Ab-FITC (AF6-120.1). Cells were incubated with 1 μg/ml bacterial LPS (Escherichia coli 055:B5) for 1 h at 37°C. Cells were washed extensively in serum-free medium before staining with Ab at a 1/200 dilution for 40 min at 4°C. Propidium iodide (PI; Sigma) was added at a 5 μg/ml final concentration, and cells were analyzed on a FACScan or FACS calibur flow cytometer (Becton Dickinson). During acquisition, cells were gated on forward scatter (FSC-H) vs PI fluorescence (FL3) to exclude dead cells. Data were analyzed by Cell Quest software.

Apoptosis assays
For apoptosis induced in the Jurkat human T cell line, effector BALB/c LPS B cell blasts were obtained at 24 h of LPS stimulation and stained with 2.5 μM 1,1′‐dioctadecyl‐3,3′,3′‐tetratetramethylindodicarbocyanine perchlorate for 15 min at 37°C. BALB/c B cell blasts were incubated with Jurkat cells at a 10:1 E:T ratio. At 6 h, the cells were washed, stained with PI, and analyzed by flow cytometry. The percentage of apoptotic cells was determined from the fraction of PI-positive cells with the gated I-Ab-positive cells comprising 0.06% of the spleen within the recipient’s spleen. On day 3 after injection vs 0.01%). However, by day 12, the two routes of injection had equalized, in that the CFSE-labeled transduced B cells comprised ~0.04% of the recipient spleen (0.5% of injected cells), regardless of route of injection. Although an i.v. route of injection seemed to maximize CFSE cell yield in the spleen at early time points (before day 12), if animals were injected with half the number of cells above (5 × 106), the CFSE populations from i.v. vs i.p. injection were comparable by day 5 after injection (data not shown).

Results
Localization of tolerogenic B cells in vivo
Tolerance induced by peptide-IgG B cell blasts has been demonstrated to persist for as long as 6–10 mo (8) (Y. Kang, R. Agarwal, R. Caspi, and D. W. Scott, unpublished observations). However, the localization and persistence of the tolerogenic B cells in vivo was unknown. To track the B cells in vivo, two methods were used. In the first, CFSE was initially used to label the cells before injection. In later studies, we used a bicistronic vector with GFP to track the cells.

As demonstrated in Fig. 1A, CFSE-labeled peptide-IgG-transduced B cell blasts comprise a small but reproducible population within the recipient’s spleen. On day 3 after i.p. injection, the CFSE-labeled transduced B cells comprised 0.06% of the spleen (1% of the initial injected population). Intravenous injection increased the CFSE cell yield in the days immediately after injection compared with i.p. injection (e.g., 0.32% of recipient spleen on day 1 after injection vs 0.01%). However, by day 12, the two routes of injection had equalized, in that the CFSE-labeled transduced B cells comprised ~0.04% of the recipient spleen (0.5% of injected cells), regardless of route of injection. Although an i.v. route of injection seemed to maximize CFSE cell yield in the spleen at early time points (before day 12), if animals were injected with half the number of cells above (5 × 106), the CFSE populations from i.v. vs i.p. injection were comparable by day 5 after injection (data not shown).
With i.p. injection, a proportion of injected cells did remain in the peritoneal cavity, peaking at 7.4% of injected cells on day 3 after injection (Fig. 1B). The numbers of CFSE-transduced B cells in lymph nodes were nominal, with the highest value being 0.1% of injected cells, detected in the mesenteric lymph nodes on day 6 after injection (data not shown). The injected cells were undetectable in peripheral blood, heart, lung, liver, or kidney.

On day 32 after injection, CFSE-labeled transduced B cells were still detectable, comprising 0.02% of the spleen (Fig. 2A). In a separate experiment, a similar population of CFSE-labeled, OVA-IgG B cells was evident on days 30 and 60 after injection (Fig. 3A). The CFSE+ cells were no longer detectable on day 120 after injection, although animals continued to exhibit T cell hyporesponsiveness to OVA at that time (Fig. 3, A and B).

**LPS blasts continue to divide in vivo**

Under the gene therapy protocol, the peptide-IgG B cells are stimulated in vitro with LPS for 48 h, washed, and injected in vivo. As indicated in Fig. 2A, the B cells continue to divide in vivo. By day 3, 64% of the peptide-IgG B cells have divided at least once; by day 7, 97% have divided at least once. The proliferation of non-transduced, LPS-stimulated B cells and OVA-IgG-transduced (LPS-stimulated) B cells is very similar (Figs. 2, B and C), suggesting that the division of the transduced B cells is a continued effect of the LPS stimulation and not due to retroviral infection. Moreover, CFSE-labeled unstimulated (resting) B cells do not divide. Thus, the observed proliferation is not due to homeostatic regulation in the recipient.

**B cells from gld mice are not tolerogenic APCs**

LPS-stimulated B cells have been reported to express up-regulated levels of FasL and to kill Fas-sensitive target cells (15–17). To examine the need for FasL on the presenting B cells, we tested B cells from gld mice, which have a mutation in FasL among other defects, for their ability to act as tolerogenic APCs. LPS-activated B cells from C57BL/6 gld or +/- mice were transduced with an Ig fusion corresponding to the C2+ A2 domain of clotting FVIII, and the B cells were transferred to syngeneic FVIII KO (E16) recipients who were then challenged with FVIII, which is known to be immunogenic under these conditions. The results presented in Fig. 4 indicate that tolerance in terms of T cell proliferation to C2 was not induced with gld B cells but did occur with +/- B cells. In addition, tolerance in terms of the Ab response to the C2 domain of FVIII was only induced in recipients of +/- B cells expressing C2-Ig compared with recipients of control B cells.

Although preliminary results in our laboratory suggested a modest increase in FasL expression on the LPS-stimulated transduced B cells (M. El-Amine and D. W. Scott, unpublished observations), these results needed to be validated both in vitro and in vivo. Indeed, we found that LPS-activated B cells exhibited small but reproducible FasL up-regulation by FACS analysis (Fig. 5A). This has been a reproducible finding that is strain independent, with a maximum of 2.5% FasL+ B cells found after 72 h in vitro with LPS (Y. Su and D. W. Scott, unpublished observations). Nevertheless, to determine whether FasL expression may continue to increase in vivo on retrovirally transduced cells, we used a bicistronic vector with GFP to track and gate these cells at 24 h after i.v. injection. However, the percentage of GFP+, FasL+ B cells in the spleen did not increase further (Y. Su and D. W. Scott, unpublished observations).

To further examine whether peptide-IgG-transduced B cells express functional FasL, direct apoptosis experiments were performed. LPS-activated B cells (for 24–72 h) were incubated with Fas-bearing Jurkat or A20 B lymphoma cells for 6 or 24 h, respectively (Fig. 5, B and C). The peptide-IgG B cells did not increase the baseline level of apoptosis exhibited by the target T or B cells, in marked contrast to the increased apoptosis caused by the anti-Fas Ab positive control or K562 effectors (FasL+). Viral transduction of LPS-activated B cells has no effect on apoptosis induction to either target T cells or B cells (Fig. 5C, top row).

**FIGURE 2.** CFSE-labeled peptide-IgG B cells proliferate in vivo. A. Normal BALB/c recipients were given i.p. injections of 5 x 10⁶ CFSE-labeled 12-26-IgG B cell blasts. On days 3, 7, and 17 after injection, two animals were killed, and spleen cells were examined by flow cytometry. Cells were stained with aCD19 PE, to delineate B cells, and with PI. One representative plot per day is shown. The plots shown were gated on the PI− CFSE+ CD19+ population. B. Normal BALB/c recipients were given i.p. injections of 5 x 10⁶ CFSE-labeled B cells, either unstimulated, LPS-stimulated for 48 h, or OVA-IgG transduced. Under the transduction protocol, B cells are stimulated with LPS for 48 h. On days 2, 5, and 8 after injection, two animals from each group were killed, and spleen cells were examined by flow cytometry. Cells were stained with aCD19 PE and with PI. Flow cytometry plots were gated on the PI− CFSE+ CD19+ population, and the percentages of CD19+ cells dividing (at least one division) were determined. Average values for each experimental group per day are shown. C. This experiment was performed as described in B. The percentages of CD19+ cells per each division on day 8 after injection were determined from the flow cytometry plots. Average values for each experimental group per day are shown.
Increasing the LPS-activated B cells/A20 cells ratio to 5:1 yielded similar results (data not shown). These data suggest that the peptide-IgG B cells do not express functional FasL.

Because we found that gld B cells express retrovirally transduced GFP-IgG protein as well as H11001/H11001/B cells (T.-C. Lei, Y. Su, and D. W. Scott, unpublished observations), therefore their failure to present for tolerance suggests that although functional FasL may be required for tolerance induction, it is more likely that gld B cells are defective at tolerogenic APCs due to other unknown defects in cells from these mice (see Discussion).

**Role of B7 expression in tolerance induction**

As the peptide-IgG B cells are stimulated with LPS for 48 h in vitro, the transduced B cells display up-regulation of both B7 co-stimulatory molecules before injection (Fig. 6). To determine whether this up-regulation is maintained in vivo, CFSE-labeled transduced B cells were monitored for B7 expression. Although B7.1 had fallen to baseline levels by day 3 after injection, B7.2 up-regulation was maintained through day 7 after injection (Fig. 6).

To determine whether B7 mediates tolerance induction in our system, two approaches were used. In the first, B cells from B7 KO donors exhibited significantly lower T cell responsiveness to the p12-26 peptide compared with control groups receiving OVA-IgG-transduced cells (Fig. 7A). In contrast, transduced B cells from B7 double KO or B7.2 single KO donors failed to induce tolerance. These data suggest that B7, particularly B7.2, contributes to tolerance induction in our system of peptide-IgG gene therapy. To confirm these studies, H-2b B7 KO mice were used with the full-length p1-102-IgG as the tolerogen. In this case, the...
H-2b-immunodominant peptide 73-88 was used for the readout. As shown in Fig. 7B, although C57BL B cell donors were tolerogenic, the double KO and the B7.2 KO B cells were not.

Finally, to establish that these results were not due to intrinsic defects of these KO mice, transduced B cells were treated with anti-B7.1 (CD80) plus anti-B7.2 (CD86) at the time of transfer to recipients; transduced B cells treated with anti-B220 were used as a control for opsonization. As shown in Fig. 7C, blocking B7 expression interfered with tolerance induction, whereas blocking B220 on these B cells did not.

Other B cell stimulators, such as anti-CD40 and CpG, also up-regulate both B7 molecules, similar to that induced by LPS. Our laboratory has previously shown that, whereas LPS- or anti-CD40-stimulated peptide-IgG B cells induce tolerance, CpG-stimulated peptide-IgG B cells do not (14). Y. Su, T.-C. Lei, and D. W. Scott, manuscript in preparation. Interestingly, in vivo, although LPS-stimulated transduced B cells maintain B7.2 up-regulation through day 10 after injection, the CpG-stimulated cells return to resting levels of B7.2 by day 3 (Fig. 8). In contrast, levels of B7.1 fall similarly in both groups, declining in both by day 3 after injection.

**Discussion**

We have shown previously that B cells that have been retrovirally transduced to express IgG fusion proteins are highly tolerogenic in vivo (4–6, 13, 17), in keeping with the established tolerogenicity of B cell Ag presentation (10–12). Moreover, we know that MHC class II but not FcR expression is necessary for this tolerogenic effect (13, 14, 18, 19). To further examine the mechanisms of this gene therapy for tolerance, we elected to follow the fate and phenotype of the transduced B cell after in vivo transfer, using CFSE...
labeling. After i.p. injection, the peptide-IgG B cells were present in the largest numbers in the peritoneum and spleen, as well as in limited numbers in the peripheral lymph nodes. The cells were undetectable in peripheral blood, heart, lung, liver, and kidney. Cells in the peritoneum likely were trapped after injection. Although some cells in this peritoneal pool probably died, a significant number of living cells remained at day 12 after injection, and cells may continue to leave the peritoneum for the secondary lymphoid organs. Within the spleen, microscopy revealed CFSE

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peptide-IgG B cells to be present in the white pulp, although it is not clear whether they are predominantly in the marginal zone (data not shown). The splenic white pulp would provide an environment for the remaining cells to continue to function.

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**FIGURE 6.** B7 expression is high on transduced B cells but wanes in vivo. Normal BALB/c recipients were given injections of 5.5 × 10^6 CFSE-labeled peptide-IgG B cells, stimulated for 48 h in vitro with 25 μg/ml LPS. On days 3, 7, and 10 after injection, three animals were killed, and spleen cells were examined by flow cytometry to determine percentage of CFSE

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cells expressing B7.

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**FIGURE 7.** B7 KO peptide-IgG B cells do not induce tolerance in vivo. A, top left, Normal BALB/c, or B7 BALB/c background double KO, B7.1

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 B cells were isolated and transduced with -IgG fusion protein as described. Normal BALB/c recipients were given injections of 3 × 10^6 p12-26-IgG (H-2d-immunodominant epitope from phage λ cI repressor)-B cell blasts or an equal number of OVA-IgG B cell blasts (control). On day 10 after injection, animals were immunized in the hind footpad with 25 μg of p12-26 peptide emulsified in CFA. Two weeks after immunization, animals were boosted i.p. with 25 μg of peptide in PBS. Fourteen days after the boost, animals were killed, and cellular responses in the spleen were assayed by [\(^{3}H\)thymidine incorporation against p12-26. B, top right, Normal C57BL/6 and C57 background B7 double KO B7.1

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, or B7.2

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 B cells were isolated and transduced with phage λ cI repressor p1-102-IgG fusion protein as described. C57BL/6 recipients were given injections of 3 × 10^6 p1-102-IgG B cell blasts or an equal number of OVA-IgG B cell blasts (control). On day 10 after injection, animals were immunized in the hind footpad with 25 μg of p1-102 emulsified in CFA. Two weeks after immunization, animals were boosted i.p. with 25 μg of protein in PBS. Fourteen days after the boost, animals were killed, and cellular responses in the spleen were assayed by [\(^{3}H\)thymidine incorporation to the H-2d-immunodominant epitope p12-26. C, bottom, Normal BALB/c B cells were isolated and transduced with p1-102-IgG fusion protein as described. BALB/c recipients were given injections of 2 × 10^6 p1-102-IgG B cell blasts or an equal number of OVA-IgG B cell blasts (control) that had been coated with a combination of anti-B7.1 and anti-B7.2 or with anti-B220. In preliminary experiments, these treatments did not alter the survival of injected, Ab-coated CFSE-labeled B cells. On day 5 after injection, animals were immunized in the hind footpad with 25 μg of p1-102 emulsified in CFA. Ten days after immunization, animals were killed, and cellular responses in the spleen were assayed by [\(^{3}H\)thymidine incorporation against the H-2d-immunodominant epitope p12-26.
in which the peptide-IgG B cells could contact and tolerate Ag-specific T cells.

CFSE⁺ peptide-IgG B cells divide in vivo, seemingly a consequence of the in vitro LPS stimulation before injection. Because CFSE-labeled, LPS-stimulated nontransduced cells divide as well, but resting B cells do not, we conclude that homeostatic regulation is not responsible for the continued division. In the spleen, the numbers of CFSE⁺ peptide-IgG B cells peaked at day 3 after injection, but a small population remained at days 30 and 60 after injection. Although CFSE⁺ peptide-IgG B cells were no longer evident in the spleen at day 120 after injection, T cell tolerance was seen in animals immunized at that time (Y. Kang and D. W. Scott, unpublished observations; R. Agarwal and R. Caspi, manuscript in preparation). This T cell hyporesponsiveness seen at 4 mo was not surprising, because studies on the kinetics of tolerance induction have shown that although B cell tolerance wanes within ~7 wk, T cell tolerance can persist for nearly 6 mo (20). Unfortunately, CFSE has limited use in determining the life span of the peptide-IgG B cells because their division dilutes the fluorescent signal. Nonetheless, it is interesting that these activated B cells do persist for >1 mo, because the life span of B cells has long been controversial (20).

LPS-stimulated B cells have been reported to express up-regulated levels of FasL and to kill Fas-sensitive target cells (15–17). However, in our system, the peptide-IgG B cell blasts only expressed low FasL, and did they kill Fas-sensitive Jurkat or A20 lymphoma cells. We reported previously that peptide-IgG-transduced B cells from gld mice did not induce tolerance (5), and we reproduce those results in this study with another Ag (Fig. 4). However, data from the Caspi laboratory suggests that such cells can mediate tolerance induction in a uveitis model, notably in another mouse strain (R. Agarwal and R. Caspi, manuscript in preparation). We suggest that the phenotype, state of activation, and behavior of gld B cells may be aberrant in gld mice: this is a more plausible explanation to explain the differences. For example, gld B show some features of activated B cells, lack expression of CD21 and CD23, and are often oligoclonal. Indeed, regulation of B7 after LPS stimulation may also be different in B cells from gld mice, because we have recently found that B7.2 is rapidly modulated in vivo on gld B cells compared with controls (Y. Su and D. W. Scott, data not shown). Thus, although the role of Fas in our system remains unclear, recent data support the premise that FasL is not required in our system of peptide-IgG gene therapy and, moreover, that deletion of target T cells is not occurring (M. T. Litzinger, Ph.D. thesis, George Washington University, and unpublished results).

We know that B7 expression on LPS-stimulated B cells is initially high. Indeed, we reported previously that anti-CTLA-4 treatment could modulate tolerance induction in our model (13), as in others (22–28). Thus, it was important to determine whether B7.1 or B7.2 was initially required for tolerance in our system. We found that transduced B cells from B7 KO donors in two model systems were not tolerogenic, suggesting an initial role for B7, particularly B7.2, in tolerance induction. Moreover, anti-B7, but not anti-B220, could block tolerance induction in this model (Fig. 7C). Intriguingly, CpG-stimulated peptide-IgG B cells, which do not mediate tolerance, exhibited faster down-regulation of B7.2 in vivo compared with LPS blasts. BALB/c B cells were purified, stimulated with LPS blasts. BALB/c recipients were given injections of 1 × 10⁷ CFSE-labeled OVA-IgG B cell blasts. On days 3, 7, and 10 after injection, three animals per group were killed, and spleens were examined by flow cytometry. Cells were stained with anti-B220, anti-CD21 and CD23, and are often oligoclonal. Indeed, regulation of B7 after LPS stimulation may also be different in B cells from gld mice, because we have recently found that B7.2 is rapidly modulated in vivo on gld B cells compared with controls (Y. Su and D. W. Scott, data not shown). Thus, although the role of Fas in our system remains unclear, recent data support the premise that FasL is not required in our system of peptide-IgG gene therapy and, moreover, that deletion of target T cells is not occurring (M. T. Litzinger, Ph.D. thesis, George Washington University, and unpublished results).

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With regard to negative signaling via CTLA-4, when the B7-high, peptide-IgG B cell blasts are injected, Ag-specific T cells in the animals would be naive and thus would not be expected to express any appreciable CTLA-4. Moreover, the peptide-IgG B cells do not appear to induce significant CTLA-4 up-regulation, because OVA-IgG B cells do not induce activation in DO11.10 OVA-specific T cells during the first 72 h in vivo, as measured by proliferation, CD69 up-regulation, CD25 up-regulation, and CTLA-4 expression (M. T. Litzinger and D. W. Scott, unpublished data). Nonetheless, CTLA-4 could be expressed at low but functionally significant levels. Although previous data from our laboratory showed that anti-CTLA-4 treatment reversed tolerance only in previously primed animals (13), the use of Ab treatment to block signaling may be less efficient than KO models.

B7 has been hypothesized to play a critical role in regulatory T cell homeostasis, as evidenced by the fact that B7 KO mice show a deficit in CD4⁺CD25⁺ T cells (28–30). Although the CD4⁺CD25⁺ population in our recipient animals does not appear to expand initially, the possibility remains that B7 expression regulates CD4⁺CD25⁺ cells to induce Ag-specific tolerance. However, because B7 expression is lost by day 10 after injection, we propose that tolerance may be maintained by a lack of the co-stimulatory second signal required for productive T cell activation. Alternatively, it is possible that basal B7 expression is sufficient to sustain a population of regulatory T cells and thus maintain tolerance (28). Indeed, recent data suggest that CD25⁺ regulatory T cells are required for and/or induced by our B cell gene therapy protocol (7, 9). Additional studies on the role of CD4⁺CD25⁺ regulatory T cells in this tolerogenic gene therapy system are under investigation.
Acknowledgments

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Disclosures

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